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(54) **PROCESS FOR PRODUCING COENZYME Q 10**

(57) The invention aims at providing a process for producing coenzyme Q₁₀ efficiently in microorganisms by utilizing a coenzyme Q₁₀ side chain synthesis gene derived from a fungal species belonging to the genus *Aspergillus* and genus *Leucosporidium*.

The present invention relates to a DNA having a

DNA sequence described under SEQ ID NO:1 and 2 or derived from the above sequence by deletion, addition, insertion and/or substitution of one or several bases and encoding a protein having decaprenyl diphosphate synthase activity.

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Description**TECHNICAL FIELD**

5 [0001] The present invention relates to a process for producing coenzyme Q₁₀, which is in use as a drug or the like. More particularly, it relates to a process for causing formation of coenzyme Q₁₀ by introducing a gene coding for a coenzyme Q₁₀ side chain synthase, which serves as a key enzyme in the biosynthesis of coenzyme Q₁₀, namely a decaprenyl diphosphate synthase into a microorganism.

10 BACKGROUND ART

[0002] An industrial process for producing coenzyme Q₁₀, which is conventional in the art, comprises, for example, isolating coenzymes of plant origin, for example of tobacco origin, and adjusting the side chain length thereof by a synthetic method.

15 [0003] It is known that coenzyme Q₁₀ is produced in a wide variety of organisms, from microorganisms, such as bacteria and yeasts, to higher animals and plants. Thus, the process comprising cultivating a microorganism and extracting this substance from cells thereof can be regarded as one of the most efficient process for producing coenzyme Q₁₀ and has actually been employed in commercial production thereof. However, the productivity of such processes can hardly be said to be good, since the yield is low and the procedure is complicated, for instance.

20 [0004] Attempts have also been made to increase the production of coenzyme Q₁₀ by isolating genes involved in the biosynthesis of coenzyme Q₁₀ and amplifying the genes utilizing the recombinant DNA technology. Coenzyme Q₁₀ is formed in vivo in a multistage process comprising complicated reactions in which a number of enzymes are involved. The route of biosynthesis thereof in prokaryotes partially differs from that in eukaryotes. Basically, however, each route comprises three fundamental steps, namely the step of synthesis of decaprenyl diphosphate, which is the source of the prenyl side chain of coenzyme Q₁₀, the step of synthesis of para-hydroxybenzoic acid, which is the source of the quinone ring, and the step of completion of coenzyme Q₁₀ through coupling of these two compounds and successive substituent conversions. Among these reactions, the reaction which determines the side chain length of coenzyme Q₁₀, namely the decaprenyl diphosphate synthase-involving reaction, which is said to be a rate-determining one in the whole biosynthetic reaction route, is considered to be the most important one.

25 [0005] Therefore, for efficient production of coenzyme Q₁₀, it is considered effective to isolate a decaprenyl diphosphate synthase gene, which is the key gene in the biosynthesis of coenzyme Q₁₀, and utilize the same for the purpose of increasing production. As for the gene source, fungi, in which coenzyme Q₁₀ is produced in relatively large amounts, are leading candidates.

30 [0006] So far, decaprenyl diphosphate synthase genes have been isolated from several microorganisms, such as *Schizosaccharomyces pombe* (JP-A-09-173076) and *Gluconobacter suboxydans* (JP-A-10-57072). However, the productivity of coenzyme Q₁₀ in these microorganisms cannot be said to be satisfactory, and the cultivation of these microorganisms and the separation/purification of coenzyme Q₁₀ therefrom have not been efficient. It has thus been desired that a microorganism-derived gene for that enzyme, which enables high level production of coenzyme Q₁₀, be isolated.

40 SUMMARY OF THE INVENTION

[0007] It is an object of the present invention to produce coenzyme Q₁₀ efficiently in microorganisms by isolating a coenzyme Q₁₀ side chain synthesis gene from a fungal species belonging to the genus *Leucosporidium* and genus *Aspergillus*, and utilizing the same to thereby solve the above-mentioned productivity problem.

45 [0008] For attaining the above object, in the present invention, firstly, investigations were made in an attempt to isolate a key gene involved in the biosynthesis of coenzyme Q₁₀, a decaprenyl diphosphate synthase gene from fungi belonging to the genus *Leucosporidium* and genus *Aspergillus* and succeeded in isolating such gene. This success has now led to completion of the present invention.

50 [0009] Thus, the present invention provides
a DNA of the following (a), (b) or (c):

(a) a DNA whose base or sequence is as described under SEQ ID NO:1;

55 (b) a DNA having a base sequence derived from the base sequence shown under SEQ ID NO:1 by deletion, addition, insertion and/or substitution of one or several bases and
encoding a protein having decaprenyl diphosphate synthase activity;

(c) a DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:1 under stringent conditions and

encoding a protein having decaprenyl diphosphate synthase activity.

[0010] The invention further provides
a DNA of the following (d), (e) or (f):

- (d) a DNA whose base sequence is as described under SEQ ID NO:2;
(e) a DNA having a DNA sequence derived from the base sequence shown under SEQ ID NO:2 by deletion, addition, insertion and/or substitution of one or several bases and encoding a protein having decaprenyl diphosphate synthase activity;
(f) a DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:2 under stringent conditions and encoding a protein having decaprenyl diphosphate synthase activity.

[0011] The invention further provides
a protein of the following (g) or (h):

- (g) a protein whose amino acid sequence is as described under SEQ ID NO:3;
(h) a protein having an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:3 by deletion, addition, insertion and/or substitution of one or several amino acid residues and having decaprenyl diphosphate synthase activity.

[0012] The invention further provides
a protein of the following (i) or (j):

- (i) a protein whose amino acid sequence is as described under SEQ ID NO:4;
(j) a protein having an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:4 by deletion, addition, insertion and/or substitution of one or several amino acid residues and having decaprenyl diphosphate synthase activity.

[0013] The invention further provides
DNAs respectively encoding the above proteins (g) to (j).

[0014] The invention further provides
expression vectors with the above DNAs inserted into vectors.

[0015] The invention further provides
transformants resulting from transformation of host microorganisms with the respective DNAs mentioned above or with the expression vectors mentioned above.

[0016] The invention still further provides
a process for producing coenzyme Q₁₀,
which comprises cultivating any of the above-mentioned transformants in a medium and recovering coenzyme Q₁₀ thus formed and accumulated in the medium.

DETAILED DISCLOSURE OF THE INVENTION

[0017] The present inventors made investigations to isolate a gene encoding the enzyme in question from fungi belonging to the genus *Leucosporidium* and genus *Asperillus*, in which coenzyme Q₁₀ is produced in relatively large amounts, and, as a result, succeeded in obtaining a fragment of the gene by the PCR method.

[0018] The sequence of a known gene encoding a decaprenyl diphosphate synthase was compared with that of a known gene encoding a polyprenyl diphosphate synthase which is an analogue of the enzyme in question and a long prenyl chain synthase of different chain length coenzyme Q, and various PCR primers were synthesized for regions showing high homology therebetween. PCR conditions were studied for various combinations of these primers and, as a result, it was revealed, by gene base sequence analysis, that when 40 PCR cycles, each comprising 94°C, 1 minute → 43°C, 2 minutes → 72°C, 2 minutes, are carried out after 3 minutes of heat treatment at 94°C, using the primers DPS-1 (SEQ ID NO:5 in the sequence listing) and DPS-1 1AS (SEQ ID NO:6 in the sequence listing), a fragment, about 220 bp in size, of the enzyme gene in question is amplified from the chromosomal gene of *Leucosporidium scotti* IFO 1212, which is a fungal species belonging to the genus *Leucosporidium* and *Aspergillus clavatus* JCM 1718, which is a fungal species belonging to the genus *Aspergillus*.

[0019] For obtaining the enzyme gene in its full length, mRNA is first prepared from a fungus body of *Leucosporidium scotti* IFO 1212 and *Aspergillus clavatus* JCM 1718 in order to respectively produce 5' terminal side sequence by

5'RACE method using a primer prepared based on the inner sequence obtained before and produce 3' terminal side sequence by conducting RT-PCR using oligo dT primer relative to a poly A sequence, which is specific for the mRNA.

[0020] The base sequence of the sequence obtained was determined, whereupon it was revealed that it has the sequence shown under SEQ ID NO:1 and NO:2 in the sequence listing. In the amino acid sequence predicted from this base sequence, there was found a sequence characteristic of decaprenyl diphosphate synthase.

[0021] The DNA of the present invention may be a DNA whose base sequence is as described under SEQ ID NO:1 or SEQ ID NO:2, or a DNA having a base sequence derived from the base sequence shown under SEQ ID NO:1 or SEQ ID NO:2 by deletion, addition, insertion and/or substitution of one or several bases and encoding a protein having decaprenyl diphosphate synthase activity, or a DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:1 or SEQ ID NO:2 under stringent conditions and encoding a protein having decaprenyl diphosphate synthase activity. A number of amino acids each may be encoded by one or more codons (genetic code degeneracy), so that a number of DNAs other than the DNA having the base sequence shown under SEQ ID NO:1 or SEQ ID NO:2 can encode the protein having the amino acid sequence shown under SEQ ID NO:3 or SEQ ID NO:4. Therefore, the DNA of the invention includes such DNAs encoding the protein having the amino acid sequence shown under SEQ ID NO:3 or SEQ ID NO:4 as well.

[0022] The expression "base sequence derived by deletion, addition, insertion and/or substitution of one or several bases" as used herein means a base sequence resulting from deletion, addition, insertion and/or substitution of such a number of bases as can be deleted, added, inserted and/or substituted according to the methods well known to those skilled in the art, for example those described in Supplemental issue, Tanpakushitsu, Kakusan, Koso (Protein, Nucleic Acid and Enzyme), PCR Method for Gene Amplification, TAKKAJ, 35 (17), 2951-3178 (1990) or Henry A. Erlich (ed.), translated into Japanese under the supervision of Ikunoshin Kato: PCR Technology (1990).

[0023] The expression "DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:1 (or SEQ ID NO:2) under stringent conditions" means a DNA obtainable by utilizing the technique of colony hybridization, plaque hybridization or southern hybridization, among others, using a DNA comprising the base sequence shown under SEQ ID NO:1 (or SEQ ID NO:2) as a probe. Those skilled in the art would be able to readily obtain the desired DNA by carrying out such hybridization according to the method described in Molecular Cloning, 2nd edition (Cold Spring Harbor Laboratory Press, 1989).

[0024] The expression "protein having decaprenyl diphosphate synthase activity" means a protein capable of synthesizing decaprenyl diphosphate in a yield of not less than 10%, preferably not less than 40%, more preferably not less than 60%, still more preferably not less than 80%, as compared with the case where a protein having the amino acid sequence shown under SEQ ID NO:3 or SEQ ID NO:4. Such capacity can be measured by reacting FPP (farnesyl diphosphate) with ^{14}C -IPP (radiolabeled isopentenyl diphosphate) in the presence of the enzyme in question, hydrolyzing the resulting ^{14}C -DPP (decaprenyl diphosphate) with phosphatase and, after separation by TLC, determining the incorporation in each spot for each chain length (Okada et al., Eur. J. Biochem., 255, 52-59).

[0025] The protein of the invention may be a protein whose amino acid sequence is as described under SEQ ID NO:3 or SEQ ID NO:4, or a protein having an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO:3 or SEQ ID NO:4 by deletion, addition, insertion and/or substitution of one or several amino acid residues and having decaprenyl diphosphate synthase activity.

[0026] "Such an amino acid sequence derived by deletion, addition, insertion and/or substitution of one or several amino acid residues" can be obtained by deleting, adding, inserting and/or substituting an amino acid residue or residues by site-specific mutagenesis or any other methods well known in the art. Such methods are specifically described, for example, in Nucleic Acid Res., 10, 6487 (1982) and Methods in Enzymology, 100, 448 (1983).

[0027] For causing expression of the decaprenyl diphosphate synthase gene, it is necessary to connect that gene to a site downstream of an appropriate promoter. It is possible to construct an expression vector, for example, by excising a DNA fragment containing the gene using a restriction enzyme or amplifying an enzyme-encoding gene portion alone by PCR and then inserting the fragment or amplification product into a promoter-containing vector.

[0028] In the practice of the invention, the vector in which a DNA encoding a protein having decaprenyl diphosphate synthase activity is to be inserted to give an expression vector is not particularly restricted but may be one derived from an *Escherichia coli*-derived plasmid with an appropriate promoter inserted therein. The *Escherichia coli*-derived plasmid includes, among others, pBR322, pBR325, pUC19, pUC18 and pUC119, and the promoter includes, among others, the T7 promoter, trp promoter, tac promoter, lac promoter, and λ PL promoter.

[0029] In the practice of the invention, pGEX-2T, pGEX-3T, pGEX-3X (the three being products of Pharmacia), pBluescriptII, pUC19, pUC18 (product of Toyobo Co., Ltd.), pMALC2, pET-3T, pUCNT (described in Publication WO 94/03613) and the like may also be used as vectors for expression. Among these, pUCNT and pUC18 are judiciously used. In specific examples, a decaprenyl diphosphate synthase gene expression vector, pNTL1, can be constructed by inserting a gene containing the DNA sequence shown under SEQ ID NO:1 into the vector for expression pUCNT, and an expression vector of decaprenyl diphosphate synthase gene, pUCA1, can be constructed when a gene containing the DNA sequence shown under SEQ ID NO:2 is inserted into the vector for expression pUC18.

[0030] And, by introducing the above enzyme gene expression vector into an appropriate microorganism, it becomes possible to utilize the microorganism for the production of coenzyme Q₁₀. The host microorganism is not particularly restricted but *Escherichia coli* is judiciously used. The *Escherichia coli* strain is not particularly restricted but includes XL1-Blue, BL-21, JM109, NM522, DH5 α , HB101, and DH5, among others. Among these, *Escherichia coli* DH5 α and JM109 are judiciously used. For example, when the decaprenyl diphosphate synthase gene expression vector pNTL1 is introduced into *Escherichia coli*, this can be transformed so that coenzyme Q₁₀, which *Escherichia coli* originally does not produce, can be produced. The *Escherichia coli* strain *E. coli* DH5 α (pNTL1) has been deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depository (zip code: 305-8566 Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) under the designation FERM BP-7353. Further, when the decaprenyl diphosphate synthase gene expression vector pUCA1 is introduced into *Escherichia coli*, this can be transformed so that coenzyme Q₁₀, which *Escherichia coli* originally does not produce, can be produced. The *Escherichia coli* strain *E. coli* JM109 (pUCA1) has been deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depository (zip code: 305-8566 Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) under the designation FERM BP-7352.

[0031] Coenzyme Q₁₀ can be produced by cultivating the transformant obtained in the invention in the conventional manner and recovering coenzyme Q₁₀ from the cultivation product. In cases where the host microorganism is *Escherichia coli*, LB medium, or M9 medium containing glucose and casamino acids can be used as the medium. For better promoter functioning, such an agent as isopropylthiogalactoside or indolyl-3-acrylic acid, for instance, may be added to the medium. The cultivation is carried out, for example, at 37°C for 17 to 24 hours, if necessary with aeration and/or agitation.

[0032] In the practice of the invention, the product coenzyme Q₁₀ obtained may be purified or used in the form of a crude product according to the selection duly made depending on the intended use thereof. Coenzyme Q₁₀ can be isolated from the cultivation product by an appropriate combination of *per se* known methods of separation and/or purification. The *per se* known methods of separation and/or purification include salting out, solvent precipitation and other methods utilizing the difference in solubility, dialysis, ultrafiltration, gel filtration, (SDS-)polyacrylamide gel electrophoresis and other methods mainly utilizing the difference in molecular weight, ion exchange chromatography and other methods utilizing the difference in charge, affinity chromatography and other methods utilizing specific affinity, reversed phase high-performance liquid chromatography and other methods utilizing the difference in hydrophobicity, isoelectric focusing and other methods utilizing the difference in isoelectric point, among others.

[0033] The field of utilization of coenzyme Q₁₀ obtained in the present invention is not particularly restricted but it may be judiciously used as a drug, among others.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034]

Fig. 1 is a restriction enzyme map of the expression vector pNTL1.

Fig. 2 is a restriction enzyme map of the expression vector pUCA1.

Fig. 3 shows HPLC analysis charts showing production of coenzyme Q₁₀ by recombinant *Escherichia coli*, *E. coli* DH5 α (pNTL1).

Fig. 4 shows HPLC analysis charts showing production of coenzyme Q₁₀ by recombinant *Escherichia coli*, *E. coli* JM109 (pUCA1).

BEST MODES FOR CARRYING OUT THE INVENTION

(Example 1)

[0035] The chromosomal DNAs of *Leucosporidium scottii* IFO 1212 and *Aspergillus clavatus* JCM 1718 were prepared by the method of C. S. Hoffman et al. (Gene, 57 (1987), 267-272). Based on the homology with the known long-chain prenol diphosphate synthase gene, primers for use in PCR, namely DPS-1 (SEQ ID NO:5 in the sequence listing) and DPS-1 1AS (SEQ ID NO:6 in the sequence listing), were designed. Using these, a PCR cycle of 94°C, 1 minute \rightarrow 43°C, 2 minutes \rightarrow 72°C, 2 minutes, were repeated 40 times after 3 minutes of heat treatment at 94°C, followed by 1.2% agarose gel electrophoresis.

[0036] The thus-obtained fragment, about 220 bp in size, was purified by excising the corresponding gel portion from the gel and then treating with a DNA extraction kit (Sephaglas (trademark) BrandPrep Kit, product of Amersham Pharmacia Biotech), and the purified fragment was cloned into a vector for expression in *Escherichia coli* using a PCR product direct cloning kit (pT7 BlueT-Vector Kit, product of NOVAGEN) to give pT7-L1DPS and pT7-A1DPS. The DNA base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer)

using a DNA sequencing kit (product of PerkinElmer, ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit with AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto. As a result, a DNA fragment having a base sequence covering the 709th to 915th bases of the base sequence shown under SEQ ID NO:1 in the sequence listing was obtained from *Leucosporidium scottii* IFO 1212, and a DNA fragment having a base sequence covering the 616th to 822nd bases of the base sequence shown under SEQ ID NO:2 in the sequence listing was obtained from *Aspergillus clavatus* JCM 1718, respectively. These DNA fragments were estimated to be part of the decaprenyl diphosphate synthase gene since the amino acid sequence "GDFLLXRA" (X is A or G), which is a characteristic region of the long-chain prenyl chain-containing prenyl diphosphate synthase, could be found in the sequence translated from the base sequence determined in the above manner.

(Example 2)

[0037] *Leucosporidium scottii* IFO 1212 and *Aspergillus clavatus* JCM 1718 were cultured in 50 mL of 703 medium (5g/L pepton, 3g/L yeast extract, 3g/L malt extract, and 1g/L glucose, pH 6.0) for 48 hours at 25°C. Then, the cells were harvested by centrifugation at 3,000 revolutions for 20 minutes and frozen immediately in liquid nitrogen.

[0038] The frozen cells were placed in a mortar chilled previously at -70°C and ground to a powder with a pestle while being kept frozen by occasionally adding liquid nitrogen. Total RNA was prepared from the well-powdered cells using an RNeasy Maxi RNA Purification Kit (product of Qiagen K.K.). Total RNA thus extracted was further purified using an RNeasy Mini RNA Purification Kit (product of Qiagen K.K.). From the purified total RNA, mRNA was prepared using an mRNA purification kit (Ologotex-dT30 <Super> (trademark) mRNA Purification kit, product of Takara Shuzo Co., Ltd.).

(Example 3)

[0039] The DNA fragment containing the region from the DNA obtained in Example 1 to the 3'-terminal region of the decaprenyl diphosphate synthase gene of *Leucosporidium scottii* IFO 1212 was obtained. RT-PCR was carried out using an RT-PCR kit (High fidelity RNA PCR Kit, product of Takara Shuzo Co., Ltd.) and primer L1S (SEQ ID NO:7 in the sequence listing), which was generated based on the internal sequence of the DNA fragment obtained in Example 1. The thus-obtained fragment, about 850 bp in size, was purified by excising the corresponding gel portion from the gel and then treating with a DNA extraction kit (Sephaglas (trademark) BrandPrep Kit, product of Amersham Pharmacia Biotech), and the purified fragment was cloned into a vector for expression in *Escherichia coli* using a PCR product direct cloning kit (pT7 BlueT-Vector Kit, product of NOVAGEN) to give pT7-L2DPS. The DNA base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer) using a DNA sequencing kit (product of PerkinElmer, ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto.

(Example 4)

[0040] The DNA fragment containing the region from the DNA obtained in Example 1 to the 5'-terminal region of the decaprenyl diphosphate synthase gene of *Leucosporidium scottii* IFO 1212 was obtained. Reverse transcription was carried out using a 5'-Full RACE Core Set (product of Takara Shuzo Co., Ltd.), the mRNA prepared in Example 2 as template, and primer L7ASP (SEQ ID NO:8 in the sequence listing, "5'-terminal region-phosphorylated"), which was generated based on the internal sequence of the DNA fragment obtained in Example 3, to synthesize a cDNA encoding the 5'-terminal region of the gene containing a part of the fragment obtained in Example 3. Then, the cDNA was circularized using the above kit.

[0041] PCR was carried out using this circularized cDNA as template, and primers L5S (SEQ ID NO:9 in the sequence listing) and L4AS (SEQ ID NO:10 in the sequence listing), which were generated based on the known part of the sequence obtained in Example 3. Furthermore, PCR was carried out to the PCR product using primers L6S (SEQ ID NO:11 in the sequence listing) and L3AS (SEQ ID NO:12 in the sequence listing) to obtain a fragment, about 950 bp in size. The thus-obtained fragment, about 950 bp in size, was purified by excising the corresponding gel portion from the gel and then treating with a DNA extraction kit (Sephaglas (trademark) BrandPrep Kit, product of Amersham Pharmacia Biotech), and the purified fragment was cloned into a vector for expression in *Escherichia coli* using a PCR product direct cloning kit (pT7 BlueT-Vector Kit, product of NOVAGEN) to give pT7-L3DPS. The DNA base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer) using a DNA sequencing kit (product of PerkinElmer, ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto.

(Example 5)

[0042] To obtain the full-length sequence of the decaprenyl diphosphate synthase gene of *Leucosporidium scottii* IFO 1212, PCR was carried out using pT7-L3DPS as template, primer LN1-2 (SEQ ID NO:13 in the sequence listing), which was generated based on the 5'-terminal sequence of the gene and primer L3AS (SEQ ID NO:12 in the sequence listing) described above to obtain the 5'-terminal region, about 700 bp in size. And, PCR was carried out using pT7-L2DPS as template, primer L1S (SEQ ID NO:7 in the sequence listing) described above and primer LCH (SEQ ID NO:14 in the sequence listing), which was generated based on the 3'-terminal sequence of the gene, to obtain the 3'-terminal region, about 770 bp in size. The two fragments were mixed and denatured, and then slowly cooled to allow annealing, followed by duplex synthesis using DNA polymerase. Subsequently, PCR was carried out using the double-stranded DNA as template, and primers LN1-2 and LCH to obtain DNA containing the full-length sequence of the gene. The base sequence was determined using a DNA sequencer (model 377, product of PerkinElmer, Inc.) and a DNA sequencing kit (product of PerkinElmer, Inc., ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto. Thus, the total sequence of the decaprenyl diphosphate synthase gene of *Leucosporidium scottii* IFO 1212 was revealed. Base sequence was determined on the DNA, about 1.5 kbp in size, and the result is shown under SEQ ID NO:1 in the sequence listing. The amino acid sequence deduced from this base sequence is shown under SEQ ID NO:3.

(Example 6)

[0043] The DNA obtained in Example 5 was cleaved with the restriction enzymes *Nde*I and *Hind*III, and the cleavage product was inserted into a vector for expression, pUCNT (described in WO 94/03613), to give a decaprenyl diphosphate synthase gene expression vector, pNTL1. The restriction enzyme map of the thus-obtained expression vector pNTL1 is shown in Fig. 1. The symbol DPS stands for the coding region of the decaprenyl diphosphate synthase gene.

(Example 7)

[0044] The DNA fragment containing the region from the DNA fragment obtained in Example 1 to the 3'-terminal region of the decaprenyl diphosphate synthase gene of *Aspergillus clavatus* JCM 1718 was obtained. RT-PCR was carried out using an RT-PCR kit (High fidelity RNA PCR Kit, product of Takara Shuzo Co., Ltd.) and primer A1S (SEQ ID NO:15 in the sequence listing), which was generated based on the internal sequence of the DNA fragment obtained in Example 1. The thus-obtained fragment, about 850 bp in size, was purified by excising the corresponding gel portion from the gel and then treating with a DNA extraction kit (Sephaglas (trademark) BrandPrep Kit, product of Amersham Pharmacia Biotech), and the purified fragment was cloned into a vector for expression in *Escherichia coli* using a PCR product direct cloning kit (pT7 BlueT-Vector Kit, product of NOVAGEN) to give pT7-A2DPS. The DNA base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer) using a DNA sequencing kit (product of PerkinElmer, Inc., ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto.

(Example 8)

[0045] The DNA fragment containing the region from the DNA fragment obtained in Example 1 to the 5'-terminal region of the decaprenyl diphosphate synthase gene of *Aspergillus clavatus* JCM 1718 was obtained. Reverse transcription was carried out using a 5'-Full RACE Core Set (product of Takara Shuzo Co., Ltd.), the mRNA prepared in Example 2 as template, and primer A7ASP (SEQ ID NO:16 in the sequence listing, "5'-terminal region-phosphorylated"), which was generated based on the internal sequence of the DNA fragment obtained in Example 7 to synthesize a cDNA encoding the 5'-terminal region of the gene containing a part of the fragment obtained in Example 7. Then, the cDNA was circularized using the above kit.

[0046] PCR was carried out using this circularized cDNA as template, and primers A5S (SEQ ID NO:17 in the sequence listing) and A2AS (SEQ ID NO:18 in the sequence listing), which were generated based on the known part of the sequence obtained in Example 3. Furthermore, PCR was carried out to the PCR product using primers A6S (SEQ ID NO:19 in the sequence listing) and A3AS (SEQ ID NO:20 in the sequence listing) to obtain a fragment, about 850 bp in size. The thus-obtained fragment was purified by excising the corresponding gel portion from the gel and then treating with a DNA extraction kit (Sephaglas (trademark) BrandPrep Kit, product of Amersham Pharmacia Biotech), and the purified fragment was cloned into a vector for expression in *Escherichia coli* using a PCR product direct cloning kit (pT7 BlueT-Vector Kit, product of NOVAGEN) to give pT7-A3DPS. The DNA base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer) using a DNA sequencing kit (product

of PerkinElmer, ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto.

(Example 9)

[0047] To obtain the full-length sequence of the decaprenyl diphosphate synthase gene of *Aspergillus clavatus* JCM 1718, PCR was carried out using pT7-A3DPS as template, primer AN2 (SEQ ID NO:21 in the sequence listing), which was generated based on the 5'-terminal sequence of the gene and primer A3AS (SEQ ID NO:20 in the sequence listing) described above to obtain the 5'-terminal region, about 700 bp in size. And, PCR was carried out using pT7-L2DPS as template, primer A1S (SEQ ID NO:15 in the sequence listing) described above and primer ACH (SEQ ID NO:22 in the sequence listing), which was generated based on the 3'-terminal sequence of the gene, to obtain the 3'-terminal region, about 770 bp in size.

[0048] The two fragments were mixed and denatured, and then slowly cooled to allow annealing, followed by duplex synthesis using DNA polymerase. Subsequently, PCR was carried out using the double-stranded DNA as template, and primers AN2 and ACH to obtain DNA. The base sequence was determined by carrying out the reaction on a DNA sequencer (model 377, product of PerkinElmer, Inc.) using a DNA sequencing kit (product of PerkinElmer, Inc., ABI PRISM (trademark) BigDye (trademark) Terminator Cycle Sequence Ready Reaction Kit With AmptiTaq (registered trademark) DNA polymerase, FS) and according to the manual attached thereto. Thus, the total sequence of the decaprenyl diphosphate synthase gene of *Aspergillus clavatus* JCM 1718 was revealed. Base sequence was determined on the DNA, about 1.4 kbp in size, and the result is shown under SEQ ID NO:2 in the sequence listing. The amino acid sequence deduced from this DNA sequence is shown under SEQ ID NO:4.

(Example 10)

[0049] The DNA obtained in Example 9 was cleaved with the restriction enzymes *Bam*HI and *Hind*III, and the cleavage product was inserted into a vector for expression, pUC18, to give a decaprenyl diphosphate synthase gene expression vector, pUCA1. The restriction enzyme map of the thus-obtained expression vector pUCA1 is shown in Fig. 2. The symbol DPS stands for the coding region of the decaprenyl diphosphate synthase gene.

(Example 11)

[0050] The decaprenyl diphosphate synthase gene expression vector pNTL1 was introduced into *Escherichia coli* DH5 α and pUCA1 was introduced into JM109, respectively, to produce recombinant *Escherichia coli*, *E. coli* DH5 α (pNTL1) and *E. coli* JM109 (pUCA1). Each recombinant *Escherichia coli* was shake-cultured overnight in 10 mL of LB medium at 37°C, and cells were harvested by centrifugation (3,000 revolutions, 20 minutes).

[0051] The cells were suspended in 1 mL of a 3% aqueous solution of sulfuric acid and, after 30 minutes of heat treatment at 120°C, 2 mL of a 14% aqueous solution of sodium hydroxide was added, followed by further 15 minutes of heat treatment at 120°C. To the thus-treated suspension was added 3 mL of hexane-isopropanol (10:2) for effecting extraction. After centrifugation, 1.5 mL of the organic solvent layer was separated, and the solvent was evaporated to dryness under reduced pressure conditions. The residue was dissolved in 200 μ L of ethanol, and 20 μ L of the solution was analyzed by high-performance liquid chromatography (using LC-10A, product of Shimadzu Corp.). For separation, a reversed phase column (YMC-pack ODS-A, 250 x 4.6 mm, S-5 μ m, 120A) was used, and the coenzyme Q₁₀ formed was detected based on the absorbance at the wavelength 275 nm. The result of *E. coli* DH5 α (pNTL1) (analyzed with mobile phase: methanol/hexane=85/15) is shown in Fig. 3, and the result of *E. coli* JM109 (pUCA1) (analyzed with mobile phase: ethanol/methanol=2/1) is shown in Fig. 4. As shown in Figs 3 and 4, it was revealed that, upon introduction of the decaprenyl diphosphate synthase gene for expression thereof, coenzyme Q₁₀, which is originally not produced in *Escherichia coli*, could now be produced in recombinant *Escherichia coli*.

[0052] The thus-obtained recombinant *Escherichia coli* DH5 α (pNTL1) (deposition/accession No. FERM BP-7353) and *E. coli* JM109(pUCA1) (deposition/accession No. FERM BP-7352) have been deposited, under the Budapest Treaty, with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depository (zip code: 305-8566 Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, JAPAN) as of November 9, 2000.

INDUSTRIAL APPLICABILITY

[0053] A gene encoding decaprenyl diphosphate synthase, which is the key enzyme in the biosynthesis of coenzyme Q₁₀, was isolated from a fungal species belonging to the genus *Leucosporidium* and genus *Aspergillus* and it was sequenced. This could successfully be introduced in *Escherichia coli* for expression thereof. Furthermore, improvements in gene sequence successfully resulted in production in significant amounts. By using the method of the inven-

tion, it becomes possible to efficiently produce coenzyme Q₁₀, which is in use as a drug, among others.

SEQUENCE LISTING

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<120> Process for producing coenzyme Q₁₀

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<150> JP2000-398658

<151> 2000-12-27

<160> 22

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<212> DNA

<213> *Leucosporidium scottii*

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Ser Ser Ser Leu Leu Gln Leu Pro Thr Glu Leu Gln Lys Leu Ser Ser
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tcc cca acc tca tcc ctc cgt cat gct tcc cct tcc cgc tcc gcc tgg 144
Ser Pro Thr Ser Ser Leu Arg His Ala Ser Pro Ser Arg Ser Ala Trp
35 40 45

act tca gcc atc ccc ggt ctc tog tct gcc acc cca ttc gct tgg act 192
Thr Ser Ala Ile Pro Gly Leu Ser Ser Ala Thr Pro Phe Ala Ser Thr
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10	gca gag gga gag aag gtc aag gat atc gcc tac tog tat ggt cgt aac			1152
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29

Claims

1. A DNA of the following (a), (b) or (c):

(a) a DNA whose base sequence is as described under SEQ ID NO:1;

(b) a DNA having a base sequence derived from the base sequence shown under SEQ ID NO: 1 by deletion, addition, insertion and/or substitution of one or several bases and encoding a protein having decaprenyl diphosphate synthase activity;

(c) a DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:1 under stringent conditions and encoding a protein having decaprenyl diphosphate synthase activity.

2. A DNA of the following (d), (e) or (f):

(d) a DNA whose base sequence is as described under SEQ ID NO:2;

(e) a DNA having a base sequence derived from the base sequence shown under SEQ ID NO:2 by deletion, addition, insertion and/or substitution of one or several bases and encoding a protein having decaprenyl diphosphate synthase activity;

(f) a DNA capable of hybridizing with a DNA comprising the base sequence shown under SEQ ID NO:2 under stringent conditions and encoding a protein having decaprenyl diphosphate synthase activity.

3. A protein of the following (g) or (h):

(g) a protein whose amino acid sequence is as described under SEQ ID NO:3;

(h) a protein having an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO: 3 by deletion, addition, insertion and/or substitution of one or several amino acid residues and having deca-

prenyl diphosphate synthase activity.

4. A protein of the following (i) or (j):

- 5 (i) a protein whose amino acid sequence is as described under SEQ ID NO:4;
 (j) a protein having an amino acid sequence derived from the amino acid sequence shown under SEQ ID NO: 4 by deletion, addition, insertion and/or substitution of one or several amino acid residues and having deca-prenyl diphosphate synthase activity.

10 5. A DNA encoding the protein according to Claim 3.

6. A DNA encoding the protein according to Claim 4.

7. An expression vector resulting from insertion of the DNA according to Claim 1 or 5 into a vector for expression.

15 8. An expression vector resulting from insertion of the DNA according to Claim 2 or 6 into a vector for expression.

9. The expression vector according to Claim 7, wherein the vector for expression is pUCNT.

20 10. The expression vector according to Claim 8, wherein the vector for expression is pUC18.

11. The expression vector according to Claim 9, which is pNTL1.

12. The expression vector according to Claim 10, which is pUCA1.

25 13. A transformant resulting from transformation of a host microorganism with the DNA according to Claim 1, 2, 5 or 6.

14. A transformant resulting from transformation of a host microorganism with the expression vector according to Claim 7, 8, 9, 10, 11 or 12.

30 15. The transformant according to Claim 13 or 14, wherein the host microorganism is a strain of *Escherichia coli*.

16. The transformant according to Claim 15, wherein the strain of *Escherichia coli* is *Escherichia coli* DH5 α or JM109.

35 17. The transformant according to Claim 16, which is *E. coli* DH5 α (pNTL1) (FRPM BP-7353).

18. The transformant according to Claim 16, which is *E. coli* JM109(pUCA1) (FRPM BP-7352).

40 19. A process for producing coenzyme Q₁₀, which comprises cultivating the transformant according to Claim 13, 14, 15, 16, 17 or 18 in a medium and recovering coenzyme Q₁₀ thus formed and accumulated in the medium.

Fig.1

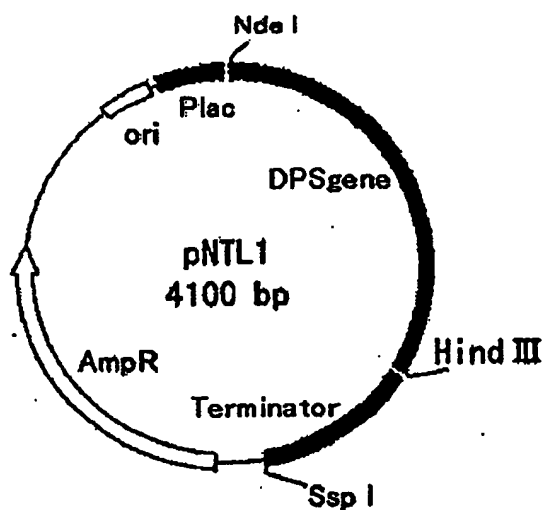


Fig.2

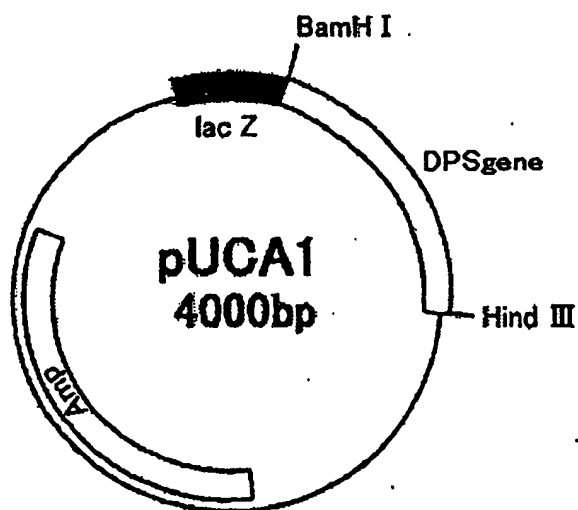


Fig.3

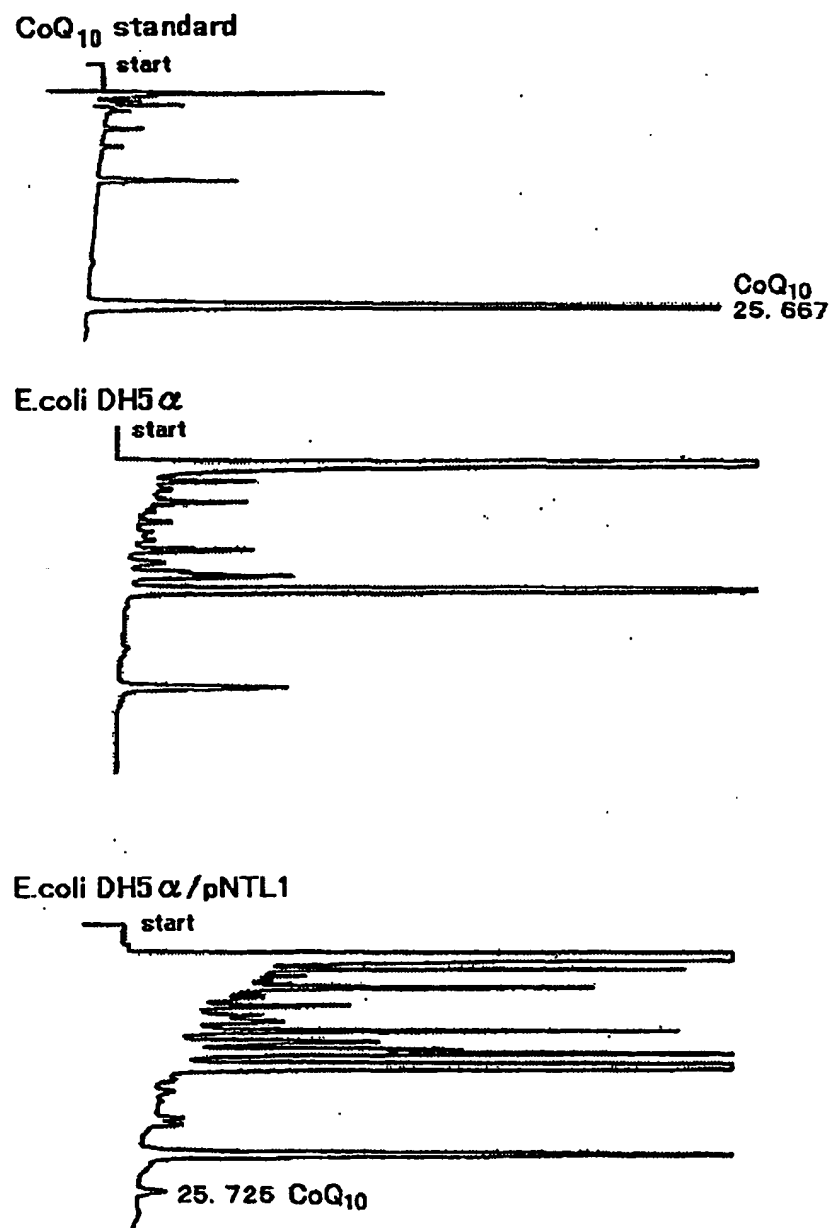
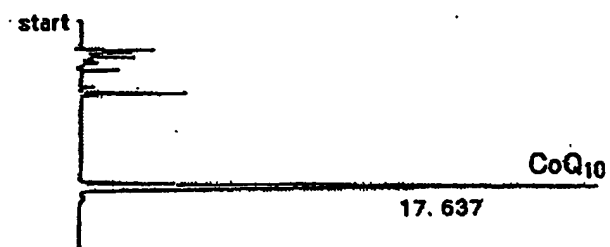
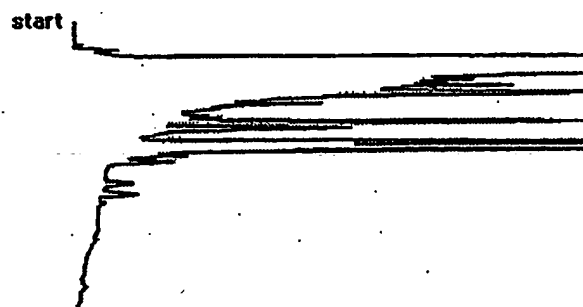


Fig.4

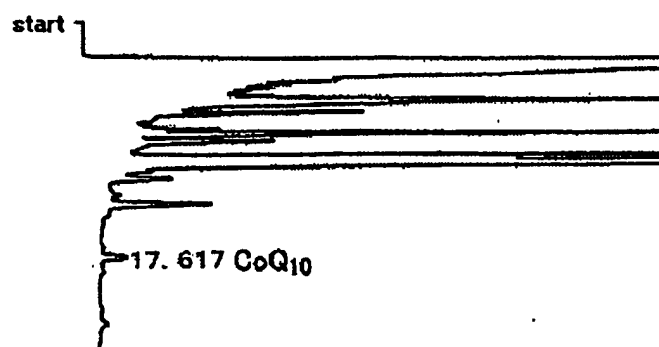
CoQ₁₀ standard



Ecoli JM109



Ecoli JM109/pUCA1



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/11523

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl⁷ C12N15/54, C12N1/21, C12P7/66, C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl⁷ C12N15/54, C12N1/21, C12P7/66, C12N9/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JICST FILE (JOIS), WPI (DIALOG), BIOSIS (DIALOG),
MEDLINE (STN), PROSITE
EMBL/DBJ/Genbank/PIR/SwissProt/Geneseq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, 6103488, A (Alpha Foods Co., Ltd.), 15 August, 2000 (15.08.00), Column 2, lines 29 to 50; Fig. 3 & JP 11-56372 A	1-19
Y	EP, 812914, A2 (Toyota Jidousha K.K.), 17 December, 1997 (17.12.97), Page 2, lines 49 to 50; page 5, line 39 to page 6, line 50; Fig. 2 & JP 10-57079 A & US 6071733 A	1-19
Y	JP, 11-178590, A (Toyota Jidousha K.K.), 06 July, 1999 (06.07.99), Page 3, Par. No. [0015], column to page 5, Par. No. [0024], column & US 6225097 B1 & US 2001-19838 A1	1-19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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means"P" document published prior to the international filing date but later
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considered novel or cannot be considered to involve an inventive
step when the document is taken alone"Y" document of particular relevance; the claimed invention cannot be
considered to involve an inventive step when the document is
combined with one or more other such documents, such
combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
07 March, 2002 (07.03.02)Date of mailing of the international search report
19 March, 2002 (19.03.02)Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/11523

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 10-57072, A (Alpha Foods Co., Ltd.), 03 March, 1998 (03.03.98), Page 3, Par. No. [0007], column to page 5, Par. No. [0008], column (Family: none)	1-19
Y	KOYAMA T. et al., Thermostable Franesyl Diphosphate Synthase of <i>Bacillus stearothermophilus</i> : Molecular Cloning, Sequence Determination, Overproduction, and Purification, J.Biochem. (1993), Vol.113, No.3, pages 355 to 363; Figs. 8, 9	1-19
Y	SHU S-O. et al., Quantative Differences of Nuclear DNA Contents and their Taxonomic Implications in <i>Leucosporidium scottii</i> , <i>Phodosporidium toruloides</i> , and Related Basidiomycetous Yeasts, J.Gen.Appl. Microbiol. (1993), Vol.39, No.3, pages 295 to 302	1,3,5,7,9,11, 13-17,19
Y	JP, 56-92793, A (MASE Tamio), 27 July, 1981 (27.07.81), Claims (Family: none)	2,4,6,8,10,12, 13-16,18-19

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